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<b>(21) International Application Number:</b> PCT/DK94/00157 <b>(22) International Filing Date:</b> 19 April 1994 (19.04.94) <b>(30) Priority Data:</b> 0445/93 20 April 1993 (20.04.93) DK <b>(71) Applicant:</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). <b>(72) Inventors:</b> SØRENSEN, Hans, Holmegaard; Joachim Rønnowsvej 21, DK-2830 Virum (DK). CHRISTENSEN, Thorkild; Bellisvej 55, DK-3450 Allerød (DK).		<b>(81) Designated States:</b> AU, BG, BR, BY, CA, CN, CZ, FI, HU, JP, KP, KR, KZ, LV, NO, NZ, PL, RO, RU, SK, UA, UZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD OF DETECTING THE PRESENCE OF AND CONVERTING OF A POLYPEPTIDE <b>(57) Abstract</b> <p>A method of detecting and treating a polypeptide. A hydrophobic derivative of a growth hormone may be detected by hydrophobic interaction chromatography and elution using a gradient of ammonium sulphate followed by peptide mapping. The derivative may be treated with a mercapto compound for converting the derivative into the native form of the growth hormone. Preferably, the growth hormone is human growth hormone, and the mercapto compound is cysteine in a concentration of 1-2 mM.</p> <p>③ A 1-10</p>		

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**TITLE**

A method of detecting the presence of and converting of a polypeptide.

**FIELD OF THE INVENTION**

5 The present invention relates to a method of detecting the presence of a hydrophobic derivative of a growth hormone and a method for converting the derivative into the native form of the growth hormone.

**BACKGROUND OF THE INVENTION**

10 The growth hormones from man and from the common domestic animals are proteins of approximately 191 amino acids, synthesized and secreted from the anterior lobe of the pituitary gland. Human growth hormone consists of 191 amino acids having a molecular weight of 22125 D. Four cystein residues  
15 are present giving rise to two disulfide bridges. The disulfide bridge formed between Cys(53) and Cys(165) results in a major loop, and the disulfide bridge between Cys(182) and Cys(189) results in a minor loop.

Growth hormone is a key hormone involved in the regulation of  
20 not only somatic growth, but also in the regulation of metabolism of proteins, carbohydrates and lipids.

The organ systems affected by growth hormone include the skeleton, connective tissue, muscles, and viscera such as liver, intestine, and kidneys.

25 Until the development of the recombinant technology and the cloning of the growth hormone gene now giving rise to production of e.g. human growth hormone (hGH) and Met-hGH in industrial scale, human growth hormone could only be obtained by extraction from the pituitary glands of human cadavers.

The very limited supplies of growth hormone restricted the use thereof to longitudinal growth promotion in childhood and puberty for treatment of dwarfism, even though it has been proposed for inter alia treatment of short stature (due to growth hormone deficiency, normal short stature and Turner syndrom), growth hormone deficiency in adults, infertility, treatment of burns, wound healing, dystrophy, bone knitting, osteoporosis, diffuse gastric bleeding, and pseudoarthrosis.

Furthermore, growth hormone has been proposed for increasing the rate of growth of domestic animals, for decreasing the proportion of fat in animals to be slaughtered for human consumption, and for increasing the production of milk in lactating animals.

In recombinant techniques human growth hormone is normally produced by expressing a gene coding for human growth hormone, said gene being inserted into a microorganism. The growth hormone is then isolated from the broth, optionally after lysing the microorganisms. The host most commonly used for expressing hGH is *E. coli*.

Growth hormone extracted from pituitary glands or growth hormone produced by recombinant techniques is always compared with suitable standards in order to ensure the identity with an authentic product.

hGH extracted from pituitaries have been investigated in order to detect aberrant forms and determine their specific activities. Besides the growth hormone with a molecular weight as mentioned above a variant single chain form is also produced, wherein the amino acid residues 32-46 are omitted resulting in the so-called 20k form of hGH. This variant is the result of alternate splicing at the m-RNA level. Also variants related to mass, charge, rearrangements, oxidized forms, and split forms are described to be present in hGH-preparations isolated from pituitary glands.

The development of new assays has enabled detection of derivatives of growth hormone present in very small amounts in preparations and standards. Thus, a hitherto unknown hydrophobic impurity has been detected in connection with the  
5 purification of human growth hormone preparations using Hydrophobic Interaction Chromatography (HIC) under special conditions. This derivative is normally not detected by any of the other methods employed for testing a sample of human growth hormone including SDS-PAGE, RP-HPLC, IE-HPLC and GPC  
10 or by the HIC method run under other conditions.

For preparing pharmaceutical preparations it is generally preferred to employ active ingredients in a form as pure as possible and, if possible, it is preferred to employ active ingredients being monocomponent compounds.

15 It is desirable to find a method for easy detection of the presence of the hydrophobic derivative of growth hormone disclosed herein as well as a need for a method for removing the derivatives from a batch of growth hormone.

It is also possible to remove the hydrophobic derivative by  
20 physical separation techniques. However, such a procedure alone is less desirable due to loss of active ingredient.

Thus there is also a need for a process which will ensure a quantitative conversion of the hydrophobic derivative of growth hormone directly into the native product.

## 25 BRIEF DESCRIPTION OF THE INVENTION

It has now been found that the hydrophobic derivative of human growth hormone disclosed herein may easily be detected by chromatographic methods and may easily be converted into the native form of human growth hormone.

Thus, in a first aspect, the invention relates to a method for detection of the presence of a hydrophobic derivative of a growth hormone comprising an extra sulphur atom as compared to the native growth hormone wherein the growth hormone is  
5 subjected to a hydrophobic interaction chromatography eluting the column using a gradient of ammonium sulphate and detecting the presence of the hydrophobic derivative.

Hydrophobic interaction chromatography is inter alia described in LC&GC.INTL Vol. 5, No. 11 (1992) 24-29.

10 The HIC may be carried out using a column of phenyl superose in a FPLC apparatus. A convenient apparatus is the FPLC apparatus Phenyl Superose HR 5/5 offered by Pharmacia.

The elution may be carried out using suitable salts such as ammonium sulphates and/or ammonium acetate.

15 The fractions of the eluate from the HIC comprising the hydrophobic derivative of growth hormone may then be subjected to peptide mapping as disclosed in Chapter 9 in High Performance Liquid Chromatography in Biotechnology, Edited by William S. Hancock, Published by John Wiley & Sons,  
20 Inc, 1990.

The hydrophobic derivative of growth hormone may be detected by comparing retention times as the fragment comprising a trisulphide bridge has a longer retention time as compared to the corresponding fragment comprising disulphide bridge.

25 In a further aspect, the present invention relates to a method for converting a hydrophobic derivative of a growth hormone into the native form of the growth hormone.

It has surprisingly been found that the hydrophobic derivative of human growth hormone may be converted into the native  
30 form thereof by treating the derivative with a mercapto

compound. The treatment is conveniently carried out in a solution comprising the hydrophobic derivative of human growth hormone in a solvent.

It has also been found that such hydrophobic derivatives may  
5 be converted directly into the native form by a gentle treatment using a mercapto compound. Thus, the conversion or "refolding" may according to the invention be carried out using a conventional buffer for refolding of proteins, but without the preceding reduction or denaturation to break the  
10 disulfide bridges normally relied upon when refolding proteins.

According to a still further aspect of the invention the hydrophobic derivative of hGH is isolated before carrying out the conversion into native hGH.

15 It is preferred to treat the whole batch of growth hormone comprising the hydrophobic derivative of hGH directly without isolating the growth hormone derivative.

The mercapto compound may be any mercapto compound not having an adverse effect on the growth hormone under the reaction  
20 conditions. Preferred compounds are such compounds which are able to transform the growth hormone derivative directly into the native form without having to reduce the growth hormone totally breaking both sulphur bridges present in native growth hormone. The mercapto compound may e.g. be cysteine,  
25 glutathione, 2-mercapto ethanol or dithiothreitol (DTT). Preferred compounds are selected from the group consisting of cysteine and glutathione. Most preferred is cysteine.

The mercapto compound is normally present in the solution in a concentration of from 0.1 and up to 5 mM. Preferably the  
30 concentration is in the interval from 0.5 to 3 mM. According to a preferred aspect of the invention, the growth hormone is treated with cysteine in a concentration of 1 to 2 mM.

In the present context "growth hormone" may be growth hormone from any origin such as avian, bovine, equine, human, ovine, porcine, salmon, trout or tuna growth hormone, preferably bovine, human or porcine growth hormone, human growth hormone  
5 being most preferred. The growth hormone to be treated in accordance with the present invention may be growth hormone isolated from a natural source, e.g. by extracting pituitary glands in a conventional manner, or a growth hormone produced by recombinant techniques, e.g. as described in E.B. Jensen  
10 and S. Carlsen in Biotech and Bioeng. 36, 1-11 (1990). The preferred growth hormone is hGH.

The "growth hormone" may also be a truncated form of growth hormone wherein one or more amino acid residues has (have) been deleted; an analogue thereof wherein one or more amino  
15 acid residues in the native molecule has (have) been substituted by another amino acid residue, preferably a natural amino acid residue, as long as the substitution does not have any adverse effect such as antigenicity or reduced action; or a derivative thereof, e.g. having an N- or C-terminal exten-  
20 sion such as Met-hGH, Met-Lys-hGH, Ala-Glu-hGH, Thr-Glu-Ala-Glu-hGH, Ala-Glu-Ala-Glu-hGH, Met-Glu-Ala-Glu-hGH, Met-Phe-Glu-Glu-hGH, Met-Asp-Ala-Asp-hGH, or Met-Glu-Ala-Asp-hGH.

The solvents used to prepare the solution of derivative of the growth hormone to be treated may e.g. be an aqueous buffer  
25 buffered at a pH from 5 to 10. Solutions being buffered to a pH > 6 are preferred. The solvent is preferably selected from the group consisting of Tris, triethylamine, citric acid, phosphate buffer, and histidine, Tris being the preferred buffer.

30 A preferred buffered solution is buffered to pH 7.5 using 20 mM Tris and 10 mM citric acid.

## DETAILED DESCRIPTION OF THE INVENTION

The identity of amino acid sequence of the hydrophobic variant of human growth hormone with that of human growth hormone has been determined by tryptic peptide mapping, amino acid sequence analysis of isolated peptide fragments. Furthermore, mass spectrometry has been carried out.

The mass spectrometry showed an increase of mass of 32 daltons of the hydrophobic derivative of hGH as compared to native hGH. This can be assigned to the presence of an extra 10 sulphur atom.

From the results of the characterization of the hydrophobic growth hormone derivative it was concluded that the derivative is a human growth hormone having one disulphide bridge (Cys 53-Cys 165) and one trisulphide bridge (Cys 182 - S - Cys 189) and having an amino acid sequence identical to that of native hGH.

## EXPERIMENTAL PART

### Example 1

#### Detection of Hydrophobic Derivative of Human Growth Hormone.

20 The presence of a hydrophobic derivative of recombinant human growth hormone comprising an extra sulphur atom as compared to the native form thereof was detected in accordance with the invention by subjecting the growth hormone to HIC using a FPLC apparatus (Pharmacia) and column of Phenyl Superose HR 25 5/5 from Pharmacia.

For elution a gradient of ammonium sulphate is used.

The buffer system was:

Buffer A: 1.2M ammonium sulphate, 20mM Tris pH 7.4

Buffer B: 20mM Tris pH 7.4

The chemicals used were all Merck p.a.

5 The elution was carried out using the following gradient:

Time (min.)	Buffer
0.0	Conc %B 0.0
1.0	Conc %B 0.0
10.0	Conc %B 100
16.0	Conc %B 100
17.0	Conc %B 0
22.0	End

The buffer was added at a rate of 0.50 ml /min, and the feed rate of the paper was 0.50 cm/min.

15 The fractions of the eluate comprising the hydrophobic derivative were subjected to peptide mapping as described above.

Alternative Method of Detection of Hydrophobic Derivative of Human Growth Hormone

20 hGH samples were analyzed on a TSK Ether 5PW (75 x 4.6 mm ID) column at ambient temperature using eluent C and D and a gradient from 40 to 50% eluent D during 30 minutes. Eluent C: 2 M  $(\text{NH}_4)_2\text{SO}_4$ , 20 mM  $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ , pH 6.0. Eluent D: 20 mM  $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ , 0.1% PEG, pH 6.0. Detection was performed at 25 280 nm. Flow: 0.5 ml/min. HPLC equipment: Data handling and control: Waters 860 Networking computer system, Pumps: Waters

pumps model 510, Sample injectors: Waters Wisp 712, Detector: Waters M481 spectrophotometer.

The hydrophobic derivative of recombinant human growth hormone (rhGH') was identified by the appearance of an new peak between peak 8 and peak 9 coupled with the disappearance of peak 7 (the 7 peptide) corresponding to amino acid residues 179-191 in a peptide mapping of recombinant human growth hormone (rhGH). The numbering of the peaks are as disclosed in Chapter 9 in High Performance Liquid  
10 Chromatographi (Supra).

#### Isolation of hydrophobic Derivative of Human Growth Hormone

If it is desired to isolate the hydrophobic derivative from a sample of hGH, such isolation may be carried out by scaling up the procedure described above, or such isolation may e.g.  
15 be carried out using the method as described in Bio/Technology 5 (1987) 161-164.

#### Characterization of Hydrophobic Derivative of Human Growth Hormone by Mass Spectroscopy

Recombinant human growth hormone was analyzed by Plasma  
20 Desorption Mass Spectroscopy (PDMS) and Electro-Spray Mass Spectroscopy (ESMS), respectively.

The analysis focused on the detecting the difference between the intact rhGH and rhGH' and the corresponding 7 and 7' tryptic peptides, respectively.

#### 25 Determination of Mass of Intact rhGH and rhGH'

The mass of intact rhGH and rhGH' was analyzed by ESMS performed using a API III LC/MS/MS system (Sciex. Thornhill, Ontario, Canada). The triple quadropole instrument had a

mass-to-charge ( $m/z$ ) range of 2400 and was fitted with a pneumatically assisted electrospray (also referred to as ion-spray) interface (P1, P1). Sample introduction was done by a syringe infusion pump (Sage Instruments, Cambridge, MA) through a fused capillary (75  $\mu\text{m}$  i.d.) with a liquid flow rate set at 0.5-1  $\mu\text{l}/\text{min}$ . The instrument  $m/z$  scale was calibrated with single charged ammonium adduct ions of poly(propylene glycols) (PPG's) under unit resolution. The accuracy of mass determination was in generally better than 0.02%, but low intensity spectra may result in less precise mass determination.

Plasma Desorption Mass Spectrometry (PDMS) analysis was performed using a BIO-ION 20K 252-Californian time-of-flight instrument (ABI Nordic A/S, Uppsala, Sweden). Standard procedures for sample application (including in situ reduction using DTT) and analysis were followed (P3,P4). The accuracy of Mass determination was about 0.1%.

Before the analysis, both rhGH and rhGH' were desalted on a Sep-pak (Stationary phase  $\text{C}_{18}$  from Waters). The rhGH' showed an increase of mass of  $31 \pm 2$  amu as compared with rhGH. After reduction using DTT, the mass of the rhGH' is identical to the calculated mass for reduced hGH.

The results are shown in the below Table I.

Table I

	ESMS	Calculated
rhGH	22126 $\pm$ 2	22125.2
rhGH'	22157 $\pm$ 2	-
rhGH' + DTT	22129 $\pm$ 2	-

Determination of Mass of the tryptic fragment No. 7 of rhGH and rhGH'

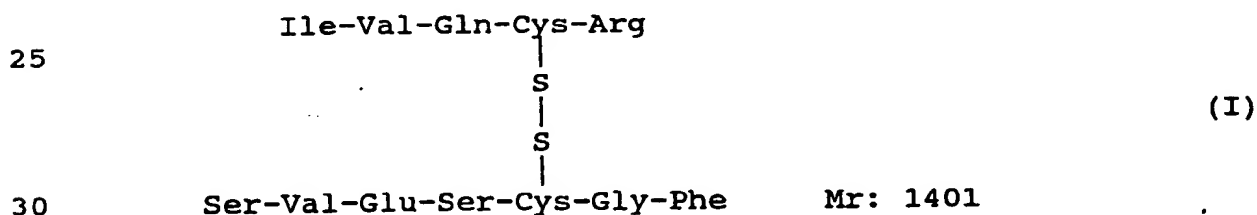
The mass of the tryptic fragment No. 7 of rhGH and rhGH', the 7 and 7' fragments, respectively, were determined by PDMS.

5 The 7 fragment arises from tryptic peptide mapping of rhGH.

hGH in a concentration of 1 mg/ml was dialysed against 50 mM Tris, 2 mM  $\text{CaCl}_2$ , 6  $\text{H}_2\text{O}$ , pH 7.8 for 24 hours at 4°C. 10  $\mu\text{l}$  of a trypsin solution prepared by dissolving 1 mg trypsin (Bovine, DPCC treated, T-1005 from Sigma) per ml. 1 mM HCl, 2 10 mM  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  was added per mg. hGH. The digestion was performed in 6 hours at 37°C. The digestion product was analysed (25  $\mu\text{l}$ ) using RP-HPLC: Column: Nucleosil RP C18, 250x4 mm, 120 Å, 7 $\mu$  (Macherey-Nagel, Art. 720042).

Temperature: 45°C. Detection : 215 nm. Flow: 1 ml/min. Eluent 15 E: 0.05% (vol/vol) TFA in water, eluent F: 0.05% (vol/vol) TFA in 70% acetonitrile in water. Gradient: 0 to 70% eluent F during 60 minutes. Then the gradient was changed to 100% F during 5 minutes followed by 10 minutes isocratic elution at 100% F. The gradient was changed back to 0% F during 1 minute 20 and the column was equilibrated for 15 minutes before next run.

The 7 fragment of rhGH produced by tryptic cleavage has a calculated mass of 1401 and the Formula I



A difference in mass of 32 amu between the 7 and 7' peptides is observed. After reduction using DTT, identical masses are found for both the 7 and the 7' peptides corresponding to the the calculated mass for the reduced peptide.

The results are shown in the below Table II.

Table II

	PDMS	Calculated
7 fragment	1401	1401
7 fragment + DTT	617 + 785	618 + 785
5 7' fragment	1433	
7' fragment + DTT	617 + 785	

The 7' fragment was isolated by collecting the fraction corresponding to the new peak by RP-HPLC of the trypsin digest as discribed above.

10 A partial Edman Degradation combined with PDMS analysis as well as ESMS was carried out directly on the 7' fragment. Through four steps it was possible to trace the manual degradation by analyzing the truncated peptide. In each step, two amino acid residues were cleaved off (one from each N-  
15 terminal). The difference in mass of 32 amu between the 7 and 7' peptides was not changed during these four cleavages.

MS/MS analysis by ESMS gave a series of ionized sequences related to the N-terminal part of the peptide. The MS/MS was carried out using the molecular ion of the 7' peptide having  
20 the mass 717amu and a double charge. The fragmentation of the "upper chain" gave rise to tops at m/z 1320, 1221, and 1094, whereas the fragmentation of the "lower chain" gave rise to tops at m/z 1247, 1118, 1061, and 974. The conclusion is that  
25 as the cystein residues - show normal masses.

Thus, the difference in mass of 32 amu between the rhGH and rhGH' seems to be due to the presence of a trisulphide as opposed to the normal disulphide.

Demonstration of the Presence of Extra Sulphur in hGH'

The presence of a trisulphide bridge was demonstrated using lead acetate as described below.

Treatment of rhGH' with cysteine as described below was demonstrated to transform the rhGH' into native rhGH during which the development of hydrogen sulphide was detected.

Filter paper (Whatman glass microfibre filters) was soaked in a 0.1M solution of lead acetate in distilled water, and air dried.

10 Six test tubes were prepared having the contents as stated in the below Table III.

Tabel III

2 tubes of 10 ml containing: Water  
 2 tubes of 10 ml containing: Pure hGH'  
 15 2 tubes of 10 ml containing: hGH - without peak 7'

The test tubes were divided into two series as stated in the below Table IV.

Tabel IV

20	<u>Series I</u>		<u>Series II</u>	
	<u>Tube</u>	<u>Containing</u>	<u>Tube</u>	<u>Containing</u>
	1	Water	4	Water
	2	Pure hGH'	5	Pure hGH'
	3	hGH (without peak 7')	6	hGH (without peak 7')

25 To all tubes of series I was added 2.5 ml of distilled water.

To all tubes of series II was added 2.5 ml of 2.5 mM cysteine in distilled water.

The paper was cut into six pieces (rondels of a diameter of 3.5 cm) and placed at the top of the eight test tubes. The 5 rondels were moistened by adding 3-4 drops of distilled water, and the test tubes were left in a water bath at 40°C for 24 hours.

After 24 hours the paper rondels were examined. No change was seen for the test tubes having had water added.

- 10 On the papers on test tubes 4 and 6 having had added cysteine, a very faint brownish colouring was observed.

The paper on test tube No. 5 showed a dense black spot ascribed to the formation of lead sulphide. The black spot appeared after 10 to 15 minutes.

15 Example 2

Conversion of Hydrophobic Derivative of Human Growth Hormone into Native Human Growth Hormone

Lyophilized rhGH from a sample comprising rhGH' was treated as follows for converting the hydrophobic derivative of hGH into native hGH:

- A: 4IU hGH were dissolved in 2.5 ml of distilled water.
- B: 4IU hGH were dissolved in 2.5 ml of distilled water followed by reduction using 100 µl mercaptoethanol for 10 minutes at ambient temperature. Then the resulting mixture was desalted using PD10 (from Pharmacia, 25 Sephadex G 25) into 20 mM Tris, pH 8.6. The solution

was left for 2h at 4°C and analyzed by hydrophobic interaction chromatography as described in Example 1.

C: 4IU hGH were dissolved in 2.5 ml of 20 mM Tris, pH 8.6. The solution was left for 2h at 4°C and analyzed by hydrophobic interaction chromatography as described in Example 1.

D: 12IU hGH were dissolved in 7.5 ml of the refolding buffer as disclosed in WO 92/03477: 20mM Tris, 2mM EDTA, 2mM Cysteine. The solution was left for 2h at 4°C and analyzed by hydrophobic interaction chromatography as described in Example 1. The sample was then desalted into 2mM His, pH 6.5 and analyzed hydrophobic interaction chromatography as described in Example 1.

15 The results show that redissolution in the folding buffer (sample D) which is weakly reducing but ensures effective disulphide formation causes transformation of the rhGH' into native rhGH.

Redissolution in water or Tris, pH 8.5 does not cause conversion (Sample A and C). If the rhGH' is completely reduced using mercaptoethanol giving a form of hGH identical to the form found in E.Coli cells (but without the presence of hydrogen disulphide in the medium) before homogenization, a correct folding may be obtained in Tris pH 8.5 without the addition of cysteine (sample B).

As shown above, rhGH' may be transformed into native hGH in the presence of 2mM cysteine. When expressing rhGH as a precursor having an amino terminal extension to be cleaved using DAP1, the cleavage may be in a medium comprising cysteine enhancing the formation of the native product having the correct disulphide bridges.

In this case the conversion and cleavage is suitably carried out in two stages, first at a high pH for converting the hydrophobic derivative whereafter the pH is lowered in order to effect the cleaving of the amino terminal extension.

5 To 4.5 ml of the eluate from the first purification step in normal purification of rhGH in 20mM Tris pH 7.5 comprising chloride ions, 10 mM citric acid at 4°C was added 0.5 ml 20mM cysteine solution in distilled water.

Samples were drawn after 1, 2, 4, 8, 16, 32 and 64 minutes, 10 desalted using a NAP-5 column (Pharmacia) according to the manufacturer's instructions eluting with 25mM Tris. After elution, the pH was adjusted to 7.5, and the contents of 7' peptide was carried out by hydrophobic interaction chromatography as described in Example 1. After 1 minute the 15 peak corresponding to the 7' protein had been reduced by ~75%, and after 4 minutes, the top had disappeared totally. Thus, the 7' protein may be converted quantitatively into the native protein by treatment with 2mM cysteine for 4 minutes at 4°C.

20 Then the pH was adjusted to 4-4.5 for cleaving off an extension, if present.

### Example 3

#### Conversion of Hydrophobic Derivative of Human Growth Hormone into Native Human Growth Hormone

25 Folding experiments were carried out with a concentration of 2 mM Cys at different pH (4.3, 6.0, 7.5). As starting material was used hGH containing hGH'. 1 ml starting material (conc. 0.7 mg/ml) was adjusted to the chosen pH and mixed with 1 ml 4 mM EDTA, 4 mM Cys, 40 mM Tris, 20 mM Citric acid. 30 At different intervals samples were withdrawn and immediately

desalted on a NAP-5 column (Pharmacia) against 20 mM Tris, 10 mM Citric acid adjusted to the chosen pH as above. HIC analysis was carried out using the first-mentioned system in Example 1.

5 The results of the experiments carried out at ambient temperatures were as follows:

The starting material had a content of hGH' of 8%.

At pH 4.3 the contents of hGH' was reduced to 7.7% after 4 minutes and a sample left overnight still had a content of 10 hGH' of 6%.

At pH 6.0 the contents of hGH' was reduced to 6.0% after 4 minutes and to 1.7% after 64 minutes. After 20 hours no hGH' was detected.

At pH 7.5 the contents of hGH' was reduced very rapidly.  
15 After 1 minute to 2.6%, after 2 minutes to 1.6%, and after 4 minutes, no hGH' was detectable.

The results of carrying out the conversion at a temperature of of 4°C and at pH 7.3 were:

After 2 minutes, the contents of hGH' was reduced to 2.5%,  
20 after 4 minutes to 1%, and after 8 minutes, no hGH' could be detected.

This shows that the conversion proceeds rapidly and quantitatively at a pH of 7.5 and more slowly and incomplete at lower pH.

25 The influence of the temperature is of minor importance.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

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(ii) TITLE OF INVENTION: A Method of Detecting the Presence of and Converting of a Polypeptide

(iii) NUMBER OF SEQUENCES: 2

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

## (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: DK 0445/93
- (B) FILING DATE: 20-APR-1993

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile Val Gln Cys Arg  
1 5

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 7 amino acids  
    (B) TYPE: amino acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Val Glu Ser Cys Gly Phe  
1                            5

**CLAIMS**

1. A method for converting a hydrophobic derivative of a growth hormone into the native form of the growth hormone, wherein the derivative of growth hormone is treated with a 5 mercapto compound.
2. A method as claimed in claim 1, wherein the mercapto compound is selected from the group consisting of cysteine and glutathione, 2-mercapto ethanol and dithiothreitol.
3. A method as claimed in claim 2, wherein the mercapto compound is cysteine.
4. A method as claimed in any of claims 1-3, wherein the concentration of the mercapto compound is up to 5 mM.
5. A method as claimed in claim 4, wherein the mercapto compound is cysteine in a concentration of from 1 to 2 mM.
- 15 6. A method as claimed in any of claims 1-5, wherein the growth hormone is human growth hormone.
- 20 7. A method for detecting the presence of a hydrophobic derivative of a growth hormone comprising an extra sulphur atom as compared to the native growth hormone wherein the growth hormone is subjected to a hydrophobic interaction chromatography and eluting the column with a salt gradient and detecting the presence of the hydrophobic derivative.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 94/00157

## A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 13/00, C07K 3/08, G01N 33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, EMBASE, WPJ, CA SEARCH, CLAIMS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9204382 (BUNGE (AUSTRALIA) PTY. LTD.), 19 March 1992 (19.03.92) --	1-6
X	WO, A1, 9002758 (PITMAN-MOORE, INC.), 22 March 1990 (22.03.90) --	1,2,4,6
A	US, A, 4985544 (YOSHIHARU YOKOO ET AL), 15 January 1991 (15.01.91) --	1-6
A	US, A, 5151501 (KEVIN M. MCCOY), 29 Sept 1992 (29.09.92) -----	1-6

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&amp;" document member of the same patent family

Date of the actual completion of the international search

8 July 1994

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28 -07- 1994

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**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

28/05/94

International application No.  
PCT/DK 94/00157

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9204382	19/03/92	AU-A- 8403591 EP-A, A- 0547102	30/03/92 23/06/93
WO-A1- 9002758	22/03/90	AU-A- 4330889 EP-A- 0433395	02/04/90 26/06/91
US-A- 4985544	15/01/91	EP-A- 0302469	08/02/89
US-A- 5151501	29/09/92	NONE	